ABSTRACT: Combination of gene therapy with tissue engineering can enhance the interplay between cells and matrix, leading to better restoration and regeneration of tissues and organs in vivo. In this study the PLGA/fibrin gel hybrids were employed to load lipofectamine/pDNA-TGF-β1 complexes and mesenchymal stem cells (MSCs) (experimental group), acting as a cartilage-mimetic tissue platform. The gene complexes distributed more evenly in the hybrid scaffolds, whereas they adhered onto the pore walls of the PLGA sponges. The filled fibrin gel rendered gene release in a slower manner, too. Moreover, the fibrin gel entrapped MSCs and contributed to a higher cell loading density in the hybrid constructs. In vivo assay showed that in the defects implanted with the experimental constructs both gene and protein expression levels of TGF-β1 were significantly higher than those of the fibrin-free group at weeks 1, 3, and 6 after surgery. The full articular cartilage defects repaired by the experimental group for 12 w were resurfaced by neo-tissues with a similar thickness, cell arrangement, and color to the normal neighboring cartilage and abundant glycosaminoglycans.

KEYWORDS: PLGA, fibrin gel, MSCs, gene therapy, cartilage repair

INTRODUCTION

Total recovery of cartilage function after damage is very hard to implement clinically due to its low cell density and the absence of blood and lymphatic vessels. Deterioration of the condition over time often leads to severe degeneration of cartilage, which is affecting millions of people in the United States. Current surgical techniques, unfortunately, often produce poor long-term outcomes such as inadequate integration between neo-tissue and native cartilage or formation of fibrocartilage. Tissue engineering and regenerative medicine offers a promising solution by selecting biomaterials as scaffolds, growth factors as effective stimulants, and chondrocytes or stem cells as cell sources.

In this strategy, it is crucial to maintain therapeutic levels of the stimulants for a long period of time to accomplish the repair of the damaged tissues. However, the recombinant growth factor may lose its effectiveness within a very short period of time owing to the unstable inherence. As an alternative, gene therapy strategy can prolong the release of therapeutic cytokines during the course of tissue repair. Generally, DNA encoding a particular protein is delivered with a vector to promote the expression of therapeutic factors. Plasmid DNA (pDNA) encoding transforming growth factor-β1 (TGF-β1) is usually chosen for cartilage repair because of its competence to induce chondrogenesis and secretion of cartilage extracellular cell matrix (ECM). Among the numerous nonviral gene delivery systems, cationic liposomes are found safe and efficient to act as the transfection reagents, which can condense DNAs into complex structures.

The lipid-based carriers have been widely investigated in the area of pharmaceutics. Many drug formulations utilizing lipid as the excipient are under clinical trials. Some lipid-based pharmaceutical products have been approved by the FDA and marketed. Although some clinical trials have already used lipid in gene therapy to treat human disease, its safety and efficacy in cartilage repair still needs to be evaluated. The traditional method utilized two-dimensional (2D) monolayer culture to testify the therapeutic effects of those transfection reagents. However, the remarkable discrepancy between the three-dimensional (3D) nature of tissues and the monolayer model may bring great uncertainty when extrapolating the in vitro findings to the in vivo situation. Therefore, 3D systems have become powerful tools for pharmaceutical reagents screening. As for the gene therapy, the 3D matrix can act...
as a reservoir for the gene agents, making the gene expression persist for prolonged time. More importantly, the key characteristics of the tissues should be recapitulated in the 3D culture system to establish the interaction mechanism among the cells, gene complexes, and matrix. Many studies have shown that the physicochemical properties of the 3D matrix have an important impact on transgene expression. For example, Shea and his colleagues found that the gene expression could be prolonged when macropores were created inside poly(ethylene glycol) hydrogels. Segura showed that soft hyaluronic acid hydrogels resulted in a higher transgene expression compared with stiff ones and that Ac-GCGWGRGDSPG-NH₂ (RGD) clustering inside hydrogels enhanced the gene expression.

Many types of scaffolds have been employed to mimic the microenvironment of natural cartilage ECM, including sponges and hydrogels fabricated from the natural and synthetic materials. In our previous study, a hybrid scaffold of fibrin gel and PLGA sponge was proposed to act as a biomimetic tissue platform for cartilage regeneration. The porous sponges own the ease for processing and can supply comparable characteristics of the tissues should be recapitulated in the 3D culture system to establish the interaction mechanism among the cells, gene complexes, and matrix. Many studies have shown that the physicochemical properties of the 3D matrix have an important impact on transgene expression. For example, Shea and his colleagues found that the gene expression could be prolonged when macropores were created inside poly(ethylene glycol) hydrogels. Segura showed that soft hyaluronic acid hydrogels resulted in a higher transgene expression compared with stiff ones and that Ac-GCGWGRGDSPG-NH₂ (RGD) clustering inside hydrogels enhanced the gene expression.

The porous sponges own the ease for processing and can supply comparable mechanical strength. The hybrids composed of sponges alone is not competent to provide inductive signals for proliferation, and the β-PLGA/MSCs/(lipofectamine/pDNA-TGF-β1) constructs were characterized in terms of lipoplex release and distribution. Finally, the performance of the PLGA/fibrin gel hybrids on the gene therapy for cartilage repair should be further studied.

Herein the PLGA/fibrin gel hybrids are chosen as a platform to evaluate the performance of lipoplexes. Mesenchymal stem cells (MSCs) are used as the cell source since they have been widely used for cartilage tissue engineering. To clarify the influence of fibrin gel in the 3D model on the gene therapeutic effect of cartilage regeneration in vivo, the experimental group is defined as PLGA/fibrin gel/MSCs/(lipofectamine/pDNA-TGF-β1) constructs, and the control group is defined as PLGA/MSCs/(lipofectamine/pDNA-TGF-β1) constructs. Beneficial effects of the PLGA/fibrin gel hybrids shall be demonstrated in terms of lipoplex release and distribution. Finally, the performance of the PLGA/fibrin gel/MSCs/(lipofectamine/pDNA-TGF-β1) in the cartilage regeneration is evaluated in vivo.

### MATERIALS AND METHODS

**Materials.** Poly(lactide-co-glycolide) (PLGA, lactide/glycolic molar ratio of 75/25, M₀ 122 kDa) was purchased from Sino Biomaterials Ltd., Changchun, China. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. Human fibrinogen (Green Cross Biological Products, Co., Ltd, Anhui, China) and bovine thrombin (CALBIOCHEM) were used for the preparation of fibrin gel.

The plasmid DNA encoding mouse-TGF-β1 (pDNA-TGF-β1) was kindly donated by Dr. Jun Liu, China Institute of Metrology. pDNA was propagated by culturing Escherichia coli in Luria–Bertani medium overnight and purified using AxyPrep Maxi Plasmid Kits (Axygen Bioscience) according to the manufacturer’s instruction. pDNA-TGF-β1 was used for the in vitro cell culture and in vivo experiments through this research.

**Preparation of PLGA/Fibrin Gel Hybrids.** The porous PLGA sponges were fabricated by a gelatin porogen-leaching method as reported previously. Those sponges were shaped into cylinders (4 mm diameter and 4 mm thickness) and sterilized by immersing in 75% ethanol for at least 4 h. Ethanol was removed by rinsing with sterile phosphate-buffered saline (PBS). Fibrinogen and thrombin were dissolved in 0.9% NaCl saline and 40 mM CaCl₂ solution, which were sterilized by filtering through syringe filters before use, respectively. After the fibrinogen solution (20 mg/mL) was loaded into the micropores of the PLGA sponge under reduced pressure, the scaffold was incubated in a thrombin solution (5 U/mL) at 37 °C for 30 min to allow the formation of fibrin gel inside the sponge. Morphology of the PLGA sponges alone and the PLGA/fibrin gel hybrid were characterized by scanning electron microscopy (SEM, JEOL JEM-200CX, Japan).

**Fabrication of PLGA/Fibrin Gel/MSCs/(Lipofectamine/pDNA-TGF-β1) Constructs.** Mesenchymal stem cells derived from rabbit bone marrow were isolated and expanded as described previously. The cells were used at passage 2 or 3. When a confluence of 80–90% was achieved, the MSCs were detached by using trypsin/ethylene diamine tetraacetate dihydrate (EDTA) solution and then centrifuged.

The gene complexes suspension was prepared at a ratio of 1:2 (DNA (μg)/lipofectamine (μL)) according to the manufacturer’s procedure, with pDNA-TGF-β1 working solution of 2 mg/mL so that the pDNA in the suspension had a concentration of 400 μg/mL.

The PLGA/fibrin gel/MSCs/(lipofectamine/pDNA-TGF-β1) constructs (experimental group) were fabricated as follows. The gene complexes suspension was mixed with fibrinogen solution (40 mg/mL) at a volume ratio of 1:1, which was used to suspend MSCs at a density of 10⁵ cells/mL. This mixture containing the MSCs was loaded into the PLGA sponge under reduced pressure and then immersed in a thrombin solution (5 U/mL) at 37 °C for 30 min for gelation.

The PLGA/MSCs/(lipofectamine/pDNA-TGF-β1) constructs without fibrin gel were set as the control group. The gene complexes suspension was mixed with a low-glucose culture medium at a volume ratio of 1:1, which was used to suspend MSCs at a density of 10⁵ cells/mL. This mixture containing MSCs was loaded into the PLGA sponge under reduced pressure.

**Distribution and Release of the Gene Complexes.** To characterize the properties of the gene complexes inside the PLGA/fibrin gel hybrids or the PLGA sponges, the samples without MSCs were prepared following the above procedures except for the use of MSCs. pDNA was replaced with sperm DNA (Sigma) to monitor the release behavior of the complexes from each group. Each PLGA/fibrin gel hybrid or PLGA sponge containing the complexes was incubated in 3 mL PBS at 37 °C. Two hundred microliters of solution was collected at predetermined time intervals, and an equal volume of PBS was supplemented. Quantitative determination of the released DNA in the medium was analyzed by a PicoGreen dsDNA assay kit (Invitrogen).

**Characterization of MSCs in Each Group.** The samples without gene complexes were prepared following the above procedures except for the use of gene complexes. For the observation of cell distribution and morphology in each group by CLSM, the cell actin and nucleus were stained. Briefly, after 5 d culture in vitro, the scaffolds were washed 3 times with PBS and then fixed in 4% paraformaldehyde at 4 °C overnight.
After 3 washes in PBS, the samples were immersed in 0.5% (v/v) Triton X-100/PBS at 4 °C for 10 min. After another 3 washes in PBS, 1% BSA/PBS was used to incubate the samples at 37 °C for 80 min to block the nonspecific interactions. Finally, the cells were stained by rhodamine phalloidin (Invitrogen) and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) for 2 h at room temperature.

In Vitro Expression of TGF-β1. The constructs of both experimental and control groups were incubated in a low-glucose culture medium containing 10% FBS and cultured at 37 °C under 5% CO2. The constructs were collected after 3, 7, 14, and 21 d and stored at −80 °C until evaluation of the expression of mouse-TGF-β1 by Western blotting (see below), respectively.

Animal Experiments. All the animal experiments were approved by the Committee on Animal Experimentation of Zhejiang University. Totally 22 rabbits were used in this study. Nineteen of them were implanted with a construct of experimental group and control group bilaterally, and 3 of them were set as normal controls for qRT-PCR as shown in Figure S2, Supporting Information. The New Zealand white rabbits (2.0–2.5 kg, four-month-old) were used for the in vivo study. The sex of the rabbits was mixed and evenly distributed within the groups. The rabbits were immobilized after administration of sodium pentobarbital solution (w/v, 3%) via ear vein with a dosage of 1 mL/kg. The knee joint was exposed by skin incision of the medial parapatellar, and then a stainless steel drill was used to create osteochondral defects (4 mm in diameter and 4 mm in depth) in the experimental and control groups. For each rabbit, each of the back knees was implanted with a construct of experimental group and control group, respectively. After 12 w transplantation, the rabbits were sacrificed by injection of excess ketamine hydrochloride, and in vivo samples (as mentioned above) were homogenized at a frozen state and then suspended in 1 mL of lysis buffer (50 mM Tris-HCl/0.1% Triton X-100, 2 mM EDTA, 100 mM NaCl, and 1 mM protease inhibitor) at 4 °C for 30 min, respectively. By centrifugation at 5000g and 4 °C for 15 min, the soluble fractions were separated from the insoluble ones. A Coomassie protein assay kit (Amresco, Solon, OH, USA) was used to measure the protein concentration in the supernatant. Forty micrograms of total proteins was loaded onto 4–20% gradient sodium dodecyl sulfate (SDS)/polyacrylamide gel (Amresco, Solon, OH, USA) for fractionation, which was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After the blots were blocked with 5% milk powder in Tris buffered saline (TBS) and incubated with specific primary antibody (mouse monoclonal TGF-β1 antibody, Santa Cruz, USA) overnight, they were developed by the secondary antibody (peroxidase-conjugated goat-anti mouse antibodies, Santa Cruz, USA), and finally incubated with the enhanced chemiluminescence substrate Super Signal West Dura (Pierce, USA). The membrane stripped and hybridized with anti-β-actin (Abcam, UK) antibody was used as a control.

Statistical Analysis. Data are expressed as mean ± standard deviation (SD). Statistical analysis is performed by the two population Student’s t test. The significant level is set as p < 0.05.

RESULTS

Scaffold Morphology. In this work the gelatin particles with a size ranging from 450 to 600 μm were used as the porogen to prepare PLGA sponges. Figure 1 shows that the
pores of a bigger size match well with those of the porogen particles. The pores of a smaller size in the pore walls were formed during the freeze-drying process as a result of phase separation (Figure 1A). The sponges have an open porosity about 85% as measured by mercury injection apparatus. The loaded fibrinogen formed a fibrin network inside the big pores with a dense texture (Figure 1B), whereas the smaller pores on the walls kept unfilled. The higher magnification image shows that the fibrin network was composed of thin fibrin fibers in a nanometer scale in diameter (Figure 1C).

**Distribution and Release of Gene Complexes.** The distribution of gene complexes inside the PLGA/fibrin gel hybrids and the PLGA sponges was characterized by CLSM (Figure 2A,B). In a wet state, the gene complexes were only found on the pore walls inside the PLGA sponges (Figure 2A). By contrast, in the PLGA/fibrin gel hybrids, the gene complexes interspersed through the whole space of the pores (Figure 2B).

Figure 2C shows that at the initial stage (12 h) more than 20% DNA was released regardless of the filling of fibrin gel. The DNAs were kept releasing from the PLGA/fibrin gel hybrids until 3 d and then leveled off. By contrast, the DNAs were released slowly and almost linearly until the end of the experimental period (15 d). At this time point, about 30% and 50% DNA was released from the PLGA/fibrin gel hybrids and PLGA sponges, respectively.

**MSCs Distribution.** After culture for 5 d in vitro the MSCs could be observed only on the pore walls in the PLGA sponges (Figure 3A,B), whereas they distributed more evenly through the whole space in the PLGA/fibrin gel hybrid (Figure 3C,D). More number of cells with cluster morphology was found inside the PLGA/fibrin gel hybrids than inside the PLGA sponges. Given that the cell distribution during the first 5 days after seeding did not change significantly, it is assumed that the cell distribution at the initial seeding stage should be the same as that shown in Figure 3.

**In Vitro and in Vivo Expression of TGF-β1.** Production of mouse-TGF-β1 inside the constructs in each group during the in vitro culture was detected by WB (Figure 4). A much higher level of TGF-β1 expression was achieved in the experimental group than in the control group at each time point. Moreover, the expressed TGF-β1 kept decreasing from 3 to 21 d in the experimental group, whereas it increased from 3 to 14 d and then declined in the control group.

Mouse-TGF-β1 expression was also measured by qRT-PCR and WB in vivo (Figure 5). Gene expression of TGF-β1 in both groups increased from 1 w to 3 w, and then declined at 6 w. At each time point, the expression levels of TGF-β1 gene in the experimental group was significantly higher than in the control group. The protein expression characterized by WB (Figure 5B) gave a similar pattern coordinating with the gene expression.

**Macroscopic View and Histological Examination.** After implantation for 12 w, the articular defects treated by the experimental constructs were resurfaced completely, and the repaired tissues had a cartilage-like macroscopic appearance,
although there were still boundaries around the defect areas (Figure 6A). By contrast, the defects treated by the control constructs were only partially resurfaced, and the central areas were still concave with a brown color (Figure 6D). The boundaries between the implantation areas and the host tissues were apparent (Figure 6D). Figure 6B,C reveals that the repaired cartilage-layer tissue in the experimental group had a similar color and thickness to the normal cartilage of the host, although a slight gap still existed. Furthermore, the cartilage layer and the subchondral bone were well separated. Figure 6E,F shows that, besides the lower height, the cell arrangement, matrix color, and morphology of the repaired tissue were apparently different from the host in the control group. The dotted line outlines the cell clusters inside the repaired matrix, which can be regarded as a sign of osteoarthritis. Figure 6B,C reveals that the repaired cartilage-layer tissue in the experimental group had a similar color and thickness to the normal cartilage of the host, although a slight gap still existed. Furthermore, the cartilage layer and the subchondral bone were well separated. Figure 6E,F shows that, besides the lower height, the cell arrangement, matrix color, and morphology of the repaired tissue were apparently different from the host in the control group. The dotted line outlines the cell clusters inside the repaired matrix, which can be regarded as a sign of osteoarthritis.39 Figure 6A,B reveals that, besides the lower height, the cell arrangement, matrix color, and morphology of the repaired tissue were apparently different from the host in the control group. The dotted line outlines the cell clusters inside the repaired matrix, which can be regarded as a sign of osteoarthritis. Figure 7A,B displays the successful deposition of GAGs in the repaired tissue in the experimental group after implantation for 12 w, which is in the same level to the neighboring normal cartilage. However, the GAGs accumulation was much less in the defects treated by the control constructs (Figure 7C,D). Additionally, Figure S1A,B, Supporting Information, displays the successful deposition of type II collagen in the repaired tissue in the experimental group after implantation for 12 w, whose amount was comparable to the neighboring normal cartilage. By contrast, the defects treated by the control constructs (Figure S1C,D, Supporting Information) show negative staining of type II collagen. Higher expression of genes of SOX9, aggrecan, and types II, X, and I collagen in the neo-formed tissue (only cartilage layer) was found in the experimental group than in the control group (Figure S2, Supporting Information). One of the samples in the

Figure 5. (A) qRT-PCR and (B) Western blotting analyses of 9 expressions of mouse TGF-β1 in full-thickness cartilage defects after being treated by the experimental and control constructs for different time, respectively. 18S rRNA was used as the internal control gene for qRT-PCR analysis. Data are expressed as mean ± SD of triplicates. *p-value < 0.05.

Figure 6. (A,D) Macroscopic photographs and (B,C,E,F) H&E staining sections of full-thickness cartilage defects after being treated by (A−C) experimental and (D−F) control groups for 12 w, respectively. (C,F) Higher magnification images at the rectangle positions of (B) and (E), respectively. Blue dotted line in (F) indicates a cell cluster.

Figure 7. PAS staining of the sections of full-thickness cartilage defects after treated by (A,B) experimental and (C,D) control groups for 12 w, respectively. (B,D) Higher magnification images at the rectangle positions of (A) and (C), respectively.
experimental group gave a very low expression level of type II collagen, leading to larger deviation of the value.

**DISCUSSION**

The PLGA/fibrin gels hybrids were used as a 3D platform in this study to evaluate the efficacy of gene therapy for cartilage repair, with pDNA-TGF-β1 as the stimulant and MSCs as the model cells. PLGA and fibrin have both been used in clinics. In particular, the fibrin can support cell growth and differentiation, sustain release of small molecules or proteins, and promote wound healing.

The constituent of the matrix has an important impact on the distribution and release behavior of gene complexes. The intensive fluorescence implies the aggregation of gene complexes on pore walls of the PLGA sponges (Figure 2A). By contrast, the gene complexes distributed more evenly in the fibrin gel-filled PLGA sponges, avoiding unfavorable accumulation (Figure 2B). Filling the PLGA sponges with the fibrin gel significantly retarded the release of DNAs for a longer period of time, too (Figure 2C). In the PLGA sponges, the gene complexes adhered onto the pore walls, and their diffusion was controlled by a concentration difference between the medium. However, in the PLGA/fibrin gel hybrids, the gene complexes were physically entrapped into the fibrin network, slowing down the release of gene complexes. Therefore, the release kinetics will largely depend on the degradation of fibrin gel. Previous results show that the fibrin gel prepared by a similar way is stable for as long as 3 weeks. Retention of the gene complexes may contribute to long time duration of target gene expression, which is necessary for the maturation of neo-formed tissues such as cartilage and subchondral bone shown in this study. A similar lipoplex distribution pattern and release behavior was also observed by Shea. They reported that the lipoplexes kept as distinct particles inside the fibrin matrix and gave a sustained release during 19 days.

The 3D matrix not only acts as a reservoir of the loaded genes but also serves as the substrate to support the cell adhesion, survival, and differentiation. Compared with the PLGA sponges, the fibrin gel inside the hybrids improved the loading density of MSCs significantly (Figure 3B,D). The fibrin gel presents as a network with a mesh size (Figure 1C) much smaller than MSCs, which protects the cells from leakage down the release of gene complexes. Therefore, the release kinetics will largely depend on the degradation of fibrin gel. Previous results show that the fibrin gel prepared by a similar way is stable for as long as 3 weeks. Retention of the gene complexes may contribute to long time duration of target gene expression, which is necessary for the maturation of neo-formed tissues such as cartilage and subchondral bone shown in this study. A similar lipoplex distribution pattern and release behavior was also observed by Shea. They reported that the lipoplexes kept as distinct particles inside the fibrin matrix and gave a sustained release during 19 days.

The fibrin gel in the hybrids improves the opportunity of cell–cell contact. It is well-known that the initiation and progression of chondrogenesis rely on the information provided by the interaction among cells. It is supposed that the MSCs residing inside the fibrin gel have a greater chance to develop direct interaction with each other than those in the PLGA sponges. Additionally, the MSCs adhered onto the pore surfaces of PLGA sponges resemble a monolayer culture, which tend to cease synthesis of GAGs and type II collagen. Both the retarded gene release kinetics and elevation of cell density facilitate the cartilage rebuilding. Histological results prove that the neo-formed tissue in the experimental group has similar tissue components and morphology to the normal cartilage (Figures 6 and 7).

The fibrin gel in the hybrids could influence chondrogenesis and the final cartilage repair outcome directly via some kinds of specific interactions, too. For example, the RGD sequences in the subunit chain of fibrin encourage the adherence of MSCs in the matrix. Fibrinogen could also interact with MSCs via CD44 in a way like the binding between hyaluronic acid and CD44. Additionally, the 3D fibrin gel network provides a more hydrophilic environment than the PLGA micropores. Interaction between MSCs and fibrin can result in the up-regulation of COL2A1, agglomeration of MSCs, and finally successful chondrogenesis of MSCs.
As an adhesion substrate, the fibrin gel has a lower mechanical strength than PLGA. It is well-known that the hardness of the substrates has a profound effect on the response behavior of MSCs to TGF-β1. Li proved that, under the stimulation of TGF-β1, MSCs differentiated into smooth muscle lineage in a stiff matrix, while those cultured in a soft matrix differentiated into chondrogenic lineage. Also, Young reported that compared to the stiff substrate, MSCs cultured on the soft substrate adopted a cell shape more favorable for chondrogenesis. Moreover, the fibrin gel matrix in the hybrids could interact with many growth factors derived from the marrow effusing during the surgical process. Localization of the growth factors can support cell proliferation after tissue injury.

**CONCLUSIONS**

The PLGA/fibrin gel hybrids were utilized as a 3D platform in this study for the evaluation of gene therapy effect in cartilage repair. Compared with the PLGA sponges, the PLGA/fibrin gel hybrids facilitated more even distribution of gene complexes in 3D space, retarded DNA release from the 3D matrix, and realized loading of MSCs with a higher density. In vitro assay showed that TGF-β1 expression in the PLGA/fibrin gel/MSCs/(lipofectamine/pDNA-TGF-β1) constructs (experimental) was much higher than that in the PLGA/MSCs/(lipofectamine/pDNA-TGF-β1) constructs (control), which was consistent with the in vivo detection. After being treated for 12 w by the experimental group, the full thickness defects of the tissue regenerate remains elusive.

**ASSOCIATED CONTENT**

1 Supporting Information

Primer sequences, immunohistochemical staining of type II collagen, and qRT-PCR analyses for the expression of chondrogenesis-related genes. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

Corresponding Authors
*(L.M.) E-mail: liema@zju.edu.cn.
*(C.G.) E-mail: cygao@mail.hz.zj.cn.

Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This research is financially supported by the National Science Foundation of China (51322302 and 51328301), the National Basic Research Program of China (2011CB606203), and the Doctoral Fund of Ministry of Education of China (20130101110124).

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